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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>5</sup> :</b>  <b>C12N 5/00</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 90/03429</b>  <b>(43) International Publication Date:</b> 5 April 1990 (05.04.90)
<b>(21) International Application Number:</b> PCT/US89/03985 <b>(22) International Filing Date:</b> 13 September 1989 (13.09.89)  <b>(30) Priority data:</b> 248,830 23 September 1988 (23.09.88) US  <b>(71) Applicant:</b> CETUS CORPORATION [US/US]; 1400 Fifty-Third Street, Emeryville, CA 94608 (US).  <b>(72) Inventors:</b> INLOW, Duane ; 630 Mariposa Avenue, 310, Oakland, CA 94610 (US). MAIORELLA, Brian ; 5661 Broadway, Oakland, CA 94618 (US).  <b>(74) Agent:</b> HALLUIN, Albert, P.; Cetus Corporation, 1400 Fifty-Third Street, Emeryville, CA 94608 (US).		<b>(81) Designated States:</b> AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).  <b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> LIPID MICROEMULSIONS FOR CULTURE MEDIA  <b>(57) Abstract</b>  Lipid microemulsions which can be added to cell culture media to provide essential lipids in a bioavailable form and their components are disclosed. Methods to disperse lipids in culture media by the use of one or more emulsifiers are described. Further disclosed are media which support growth of cells and production of recombinant, viral and/or native products wherein lipids are supplied in the form of microemulsion.		

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## 5                   LIPID MICROEMULSIONS FOR CULTURE MEDIA

This invention is in the field of fermentation and cell culture. More particularly, the invention concerns cell culture media wherein lipids are supplied in the form of microemulsions and methods to disperse lipids in cell culture media.

Lipids are an apparently essential requirement for most animal cell culture.

10 [See Yamane et al., Proc. Japan Acad. 57B(10):385-389 at p. 388 (1981); and Iscove, "Culture of Lymphocytes and Hemopoietic Cells in Serum-Free Medium," In Barnes et al. (eds.), Methods for Serum-Free Culture of Neuronal and Lymphoid Cells, pp. 169-185 (1984), where it is stated at page 170: "If the experience with lymphocytes and hemopoietic cells can be extrapolated, lipids may turn out to be a universal  
15 requirement for freshly explanted cells."] The direct addition of lipids to media is not practical due to their low solubility. Conventionally, in serum-containing media, lipids are provided to the medium in the serum wherein the lipids are carried as water soluble lipoproteins; whereas conventionally, in serum-free media, lipids are carried by albumin to which the lipid is coupled through a process employing a  
20 solvent, such as chloroform. [Goodwin et al., In Kurstak et al., (eds.), Invertebrate Systems In Vitro, pp. 493-508 (1980); Iscove, id.].

Although conventional culture media have been generally prepared by adding serum in large amounts (about 5 to 20%), the use of serum has a number of disadvantages including (1) expense, in that, more than half of the cost of the media  
25 is usually occupied by the costs of the sera; (2) time necessary to check quality of sera which may vary from lot to lot and can be contaminated with mycoplasma or virus; and (3) downstream purification problems, in that, sera can contain various unidentified proteins. Similar considerations pertain to the use of albumin, a major plasma protein in conventional serum-free media. [See Yamane et al., Proc. Japan Acad., 57B(10):385-389 (1981) wherein it is stated at page 388: "BSA is, needless  
30 to say, a serum protein which is often discrepant in its cell growth promoting action of each commercial preparation."] As it is difficult to purify albumin on a large scale, commercially available albumin preparations can contain other contamination proteins, and not even crystallized preparations exceed 98% purity. "In addition to  
35 contaminating proteins, albumin preparations can be presumed to contain other

hydrophobic substances including fatty acids and steroid hormones, and traces of salts and buffers used in the purification along with their contaminating transition metal ions." Iscove, *id.* at page 176. Therefore, it would be advantageous to eliminate from cell culture media both serum and albumin, as well as other proteins as lipid carriers. This invention provides the option of eliminating such lipid carriers.

Attempts have been described in the literature to supply lipids as liposomes to cells in culture (1,2,3). These liposomes are most typically prepared by sonicating the lipid mix in the presence of a protein (albumin) and result in particles of size typically 25 nm (4).

10 Iscove, *id.* at page 179-181, provides an example of the prior art process used to supply lipids to serum-free culture. In such a process, lipids are mixed by being dissolved in chloroform; the chloroform is then evaporated and the lipids are "dried onto the bottom of the beaker." A suspending medium containing albumin is then added and the mixture is sonicated to disperse the lipids in the form of vesicles--  
15 liposomes small enough to pass through the pores of a sterilizing filter (0.45  $\mu$ m). Iscove's following description of such a process points out some of the inherent problems of supplying lipids to culture medium in such a manner:

At the end of the sonication period, a few small aggregates of soybean lipid may have escaped dispersion. These are ignored. The  
20 suspension is passed once through a membrane of 1.2- $\mu$ m pore size... after which it usually passes without difficulty through a sterilizing 0.45- $\mu$ m filter. If the filter blocks rapidly, it may be helpful to pass the suspension once more through a 1.2- $\mu$ m membrane. The grey residue on the filter is particulate metal shredded from the sonicator probe.  
25 Filtration is easiest immediately after sonication and should not be delayed....

The resulting suspensions are stored at 4°C. They may become somewhat more opaque in the first day or two of storage. This change probably reflects some coalescence of the liposomes to larger average  
30 size but has no influence on their effectiveness in culture....

Because of quantitative variation in the completeness of dispersion and retention on the sterilizing filters, each individual lipid preparation should be titrated in culture to determine its optimal concentration....

Filter-sterilization of medium which has already been supplemented with lipid should be avoided, since there is a risk of retaining an indeterminate amount of lipid on the filter. [Emphasis added.]

The introduction of lipids to cell culture media according to this invention  
5 enhances the availability of lipids to the cells and avoids globular lipid aggregates that can be lost in filtration and thus become unavailable to the cells. This invention further provides the option of not having to filter sterilize the lipid component and the rest of the media components separately.

This invention concerns stable lipid microemulsions which can be added to  
10 cell culture media to provide essential lipids in a bioavailable form. The invention further concerns cell culture media in which lipids are supplied in the form of such lipid microemulsions.

This invention further provides for methods to disperse lipids in culture media by the use of one or more emulsifiers. The emulsifiers are preferably phospholipids  
15 and/or non-toxic polymeric detergents, and still more preferably lecithin and/or Pluronic polyols and/or polysorbate compounds.

This invention further provides for a lipid solution component comprising lipids, an organic solvent and one or more emulsifiers, and an aqueous component comprising one or more emulsifiers and water. It is the addition with agitation as by  
20 vortexing of the aqueous component to the lipid solution component which results in the formation of a lipid microemulsion.

Figure 1 shows the growth of Sf9 cells in the presence or absence of lipid supplement.

Figure 2 shows the particle size diameter profile of the lipid emulsion.

25 The introduction of lipids in the form of microemulsion enhances the availability of the lipids in the media to the cells. A microemulsion is herein defined as a preparation wherein one liquid is distributed in small globules throughout the body of a second liquid wherein said small globules are herein described as micelles. The lipid microemulsions of this invention are prepared by combining with agitation  
30 an aqueous component and a lipid solution component, wherein the aqueous component is herein defined as comprising one or more emulsifiers in water, and the lipid solution component is herein defined as comprising one or more lipids and one or more emulsifiers in an organic solvent.

As indicated above, the lipid microemulsions of this invention provide a means of eliminating lipid carriers such as serum, serum albumin, or other proteins from culture media. Within the scope of this invention are also media which correspond to conventional culture media except that the serum moiety is partially replaced with at least one lipid nutrient in the form of a microemulsion of this invention. Therefore, serum or other proteinaceous substances such as albumin of conventional media can be reduced or dispensed with according to this invention. Use of the culture media of this invention makes it easier to isolate and purify the product accumulated in the media. Mass production of culture media is also made easier, according to this invention.

Another advantage of this invention is that preferred emulsifiers of this invention, Pluronic polyols, are already used as physical protectants in mammalian cultures, and have been shown therein to be non-toxic to cells and non-inhibitory to cell growth. Kilburn et al., Biotech. & Bioengin., Vol X:801-814 (1968); Mizrahi, Develop. Biol. Standard, 55:93-102 (1984); and Mizrahi, J. Clin. Microbiol., 2(1):11-13 (July 1975). PCT US 89/01029, published February 9, 1989 and PCT US 89/01028, published February 9, 1989 provide examples of successful cultivation of insect cells in media containing Pluronic polyols and the expression of recombinant proteins by such insect cells. PCT US 89/01028, supra, describes the use of lipid microemulsions herein disclosed in serum-free, low protein or essentially no protein media for insect cell culture. PCT US 89/01029, supra, discloses the successful culturing of insect cells in airlift fermentors.

Thus, Pluronic polyols, preferred emulsifiers of this invention, serve dual purpose in providing not only a physical protectant for agitated and/or sparged cultures but also in providing an emulsifying system for lipids to be supplied to the culture. Therefore, media of this invention provide an enhanced nutritional environment for the growth of cells under any culture conditions but especially for cells growth under agitated and/or sparged conditions, and, in general, for cultures that are well-aerated. As a consequence, the media thus provide an enhanced nutritional environment for the production of recombinant, viral and/or natural products, wherein examples of natural products are native proteins or monoclonal antibodies.

The term "protein" is herein defined to exclude the term "peptones." The

media of this invention preferably contain very low concentrations of protein, that is, preferably less than about 1000  $\mu$ /ml, more preferably less than about 50  $\mu$ g/ml, still more preferably less than about 5  $\mu$ /ml. If certain optional components are determined to be desirable as indicated below, for example, growth factors such as

5 insulin, transferrin or catalase, they are present preferably at low concentrations and do not function as lipid carriers.

Lipids are defined herein as any of a heterogeneous group of fats and fatlike substances characterized by being water-insoluble and being extractable by nonpolar (or fat) solvents such as alcohol ether, chloroform or benzene, among others. Lipids

10 include unsaturated fatty acids, saturated acids, steroids, lipid soluble vitamins and phospholipids and their esters. Examples of unsaturated fatty acids include linoleic acid, linolenic acid, oleic acid, arachidonic acid, and uric acid and their glycerides and esters. Preferred fatty acids also include fatty acid esters, preferably polyunsaturated fatty acid esters, and still more preferably, mixtures of

15 polyunsaturated fatty acid methyl esters, as for example, those found in fish liver oil, preferably cod liver oil. Examples of saturated fatty acids include palmitic acid and stearic acid and their glycerides. Examples of lipid soluble vitamins include vitamins A, D and E. Examples of steroids, that is lipids which contain a hydrogenated cyclopentophenanthrene-ring system, include progesterone, adrenocortical hormones,

20 the gonadal hormones, cardiac aglycones, bile acids and sterols (such as cholesterol). Preferred steroids are sterols, preferably cholesterol.

The type of lipids to be supplied to cell culture media of this invention depend on the choice of cells to be cultured. Also, lipids are supplied in concentrations appropriate for the particular cell line being cultured, wherein the

25 concentration is not toxic to the cells and is non-inhibitory to cell growth. For example, insect cells appear to be able to tolerate a higher concentration of lipids than mammalian cells. Exemplary lipid components for a mammalian cell culture are shown in Examples 1 and 2, below, whereas an exemplary lipid component of

Example 1 for the mammalian cells is shown in Example 3. The lipid component of

30 Example 1 for the mammalian cells is the same as that for the insect cells of Example 3 except in the respect that the concentrations of the lipid and the emulsifier Tween 80 are ten times less for the mammalian lipid component than for the insect lipid component. Preferred ranges for lipids for insect cells are as follows:

A mixture of polyunsaturated fatty acid methyl esters, such as, fish liver oil, preferably cod liver oil, is preferably present in the media at a concentration of from about 1 mg/l to about 50 mg/l, preferably from about 5 mg/l to about 15 mg/l, and most preferably about 10 mg/l. Said concentrations of cod liver oil further contain the preferred concentrations of the lipid soluble vitamin A. The sterol, preferably cholesterol, is at a concentration from about 2 mg/l to about 7 mg/l, more preferably from about 3 mg/l to about 5 mg/l, and most preferably about 4.5 mg/l. The alpha-tocopherol of the lipid component is at a concentration of from about 0.5 mg/l to about 4 mg/l, more preferably about 2 mg/l.

Preferred examples of insect cell culture media containing the lipid microemulsions of this invention are those disclosed in claimed in U.S. Serial No. 077,303. The compositions of three of such preferred serum-free, low protein or essentially no protein media for insect cell culture used in the examples below are outlined in Table 1. The Pluronic Polyol-Lipid Microemulsions in Table 1 are prepared according to Example 3, infra.



TABLE 1Serum-Free Insect Media Containing Lipid Microemulsions

		DFM With No Bovine Serum <u>Albumin (BSA)</u>	<u>ISFM-3</u>	<u>ISFM-4</u>
5	<b><u>IPL-041 Basal Medium</u></b>	<b><u>Yes</u></b>	<b><u>Yes</u></b>	<b><u>Yes</u></b>
	<b><u>Ultrafiltered Peptones</u></b>			
	Tryptose Phosphate Broth	2.6 g/l	No	No
	Bactocasitone (caseine digest)	1 g/l	No	No
10	Panmede (ox liver digest)	1 g/l	No	No
	Yeastolate (yeast extract)	1 g/l	2 g/l	4 g/l
	Lactalbumin Hydrolyzate	No	2 g/l	No
	<b><u>Extra Water Soluble Ingredients</u></b>			
	$\alpha$ -Glycerol-phosphate	1 g/l	No	No
15	Glycerol	2 g/l	No	No
	Folic Acid	3.6 mg/l	No	No
	Inositol	10 mg/l	No	No
	Catalase	3 mg/l	No	No
	<b><u>Pluronic Polyol-Lipid Microemulsion</u></b>			
20	Pluronic Polyol F68 in Water	(0.1%)	1 g/l	1 g/l
	Cod Liver Oil	10 mg/l	10 mg/l	10 mg/l
	Tween 80	25 mg/l	25 mg/l	25 g/l
	Cholesterol	4.5 mg/l	4.5 mg/l	4.5mg/l
	$\alpha$ -Tocopherol acetate	2 mg/l	2 mg/l	2 mg/l
25	Ethanol	1 ml/l	1 ml/l	1 ml/l

An advantage of lipids being supplied in the form of a microemulsion, in addition to enhancing the availability of the lipids to the insect cells, is in providing the option of not having to filter sterilize the lipid fraction and the rest of the media components separately. The lipid microemulsions of this invention can be added to the media without being filter sterilized, and the entire media can then be filter sterilized without the concern of lipid loss during the filter sterilization process. For large scale production, such an advantage is significant. However, it is of course possible to filter sterilize the lipid solution component and aqueous component separately and prepare the lipid microemulsion aseptically.

The lipid solution component of the media of this invention is prepared by combining the appropriate amount of a mixture of lipids, for example, a mixture of polyunsaturated fatty acids, alpha-tocopherol acetate and cholesterol, and the

emulsifier(s), in an appropriate amount of an organic solvent, preferably a C<sub>1</sub>-C<sub>3</sub> alcohol, more preferably ethanol, to form a solution. It is important that the final concentration of the organic solvent is non-toxic and noninhibitory to cell growth. For example, a preferred concentration of ethanol as the organic solvent in preferred  
5 media of this invention would be approximately 1 ml/l. The lipid solution component is then optionally filter sterilized. (The lipid solution component can be stored under nitrogen in a temperature range of from about -20°C to about -80°C.)

The aqueous component containing one or more emulsifiers, preferably protective agent/emulsifiers, is similarly optionally filter sterilized. The aqueous  
10 component, which is preferably of greater volume than the lipid solution than the lipid component is slowly and, optionally aseptically, added to the lipid solution component with agitation as by vortexing. The lipid microemulsion is formed upon the addition with agitation of the aqueous component to the lipid solution component. (In general, it is preferred to add the aqueous component to the lipid solution  
15 component rather than vice versa.)

The optionally filter sterilized lipid microemulsion can then be added to the media. If the option of aseptically forming the lipid microemulsion from filter sterilized components is not taken, the option remains of filter sterilizing the entire medium after all additions are completed.

20 Emulsifiers preferred for preparing the lipid microemulsions of this invention are non-toxic polymeric detergents, bile salts, such as, deoxycholate, and/or phospholipids. Preferably, the emulsifiers are non-toxic polymeric detergents and/or phospholipids. It is further preferred that the non-toxic polymeric detergents are non-ionic and that the phospholipid emulsifier is lecithin. It is further preferred that there  
25 be one or more emulsifiers present in both the aqueous and lipid solution components.

Editions of McCutcheon's Emulsifiers & Detergents (published by the McCutcheon Division of MC Publishing Co., 175 Rock Road, Glenn Rock, NJ, U.S.A.) are examples of a source of finding non-toxic, non-ionic polymeric detergent  
30 candidates for use as emulsifiers of this invention. *Infra*, a simple test is outlined for selecting appropriate emulsifiers within the scope of this invention.

The emulsifiers are present at final concentrations in the media which are non-toxic to cells and noninhibitory to cell growth, reproduction and expression of

products thereby. The emulsifiers are considerably more concentrated in the aqueous and lipid solution components than in the media in which they are diluted, preferably by a factor of 100 or 1000, respectively. For example, 10% Pluronic F68 in 10 ml of aqueous component and 25 mg of Tween 80 in 1 ml of lipid solution component  
5 once combined with agitation to form a lipid microemulsion which is then added to one liter of media are respectively diluted to concentrations of 0.1% (100 fold dilution for Pluronic F68) and to 25 mg/l (1000 fold dilution for Tween 80).

Very occasionally, the solubility limits of the volumes of the aqueous or lipid solution components could be reached before the maximum, non-toxic and beneficial  
10 concentration of a particular emulsifier has been reached for the medium as a whole. If such an event should occur, the emulsifier could be added directly to the medium, that is, outside of the lipid microemulsion, if it is determined that such an addition would be beneficial to the cells, as, for example, by enhancing the protective and/or emulsifying effects of a protective agent/emulsifier. Therefore, a preferred  
15 concentration (weight/volume) range for Pluronic polyols in the media of this invention would be from about 0.01% to about 1%; however, the solubility limit of Pluronic F68 is about 10% in water, and therefore, a 10 ml aqueous component would not be sufficient to accommodate the high end of the emulsifier concentration range in the final media. Additional Pluronic F68 could thus be added directly to the  
20 media if such additions would be considered necessary to optimize the protective/emulsifying effects of said protective agent/emulsifier. The concentration of Pluronic F68 in a 10 ml aqueous component would be preferably from about 1% to about 10%, more preferably from about 5% to about 10%, and still more preferably about 10%.

25 It is preferred that the emulsifiers of the aqueous component be protective agents. Protective agents are herein defined as non-toxic, water soluble compounds that functionally act to protect cells from damage and death in agitated and/or sparged cultures.

A protective agent candidate can be selected by first confirming that it is non-  
30 toxic to the cells to be cultured by methods known to those skilled in the art of cell culture, for example, by adding it to a suspension or monolayer of the cells of choice for cultivation and comparing the growth of the culture to a control. Then, the non-toxic protective agent candidates can be tested for protective ability by adding the

candidate agent to an agitated and/or sparged culture of the cells of choice at small scale and observing viability and growth rate after an appropriate period and comparing the viability and growth rate of the cells of said culture to the viability and growth rate of the cells in a control culture. A source of finding non-toxic, non-ionic polymeric detergent protective agent/emulsifier candidates for the media of this invention can be found in Editions of McCutcheon's Emulsifiers & Detergents, supra.

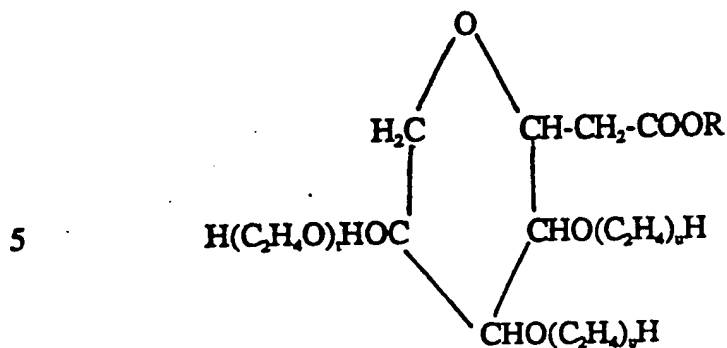
It is further preferred that the emulsifier or emulsifiers of the aqueous component block copolymers of propylene oxide and ethylene oxide (polyoxypropylene polyoxyethylene condensates). Preferably such protective agent/emulsifiers are Pluronic polyol, such as, Pluronic F68, F77, F88 and F108, preferably F68 and F88, more preferably F68. The Pluronic polyols are commercially available from BASF Wyandotte Corp. (101 Cherry Hill Road, P.O. Box 181, Parsippany, NJ 07054, U.S.A.).

The concentration of the protective agent/emulsifier(s) is preferably that which is most effective in protecting the cells from damage, but which concentration is non-inhibitory to cell growth, reproduction and expression of products thereby. The Pluronic polyol protective agent/emulsifiers are present in the media of this invention preferably at a concentration (weight/volume) of from about .01% to about 1%, more preferably from about .05% to about 0.5%, and most preferably about 0.1%.

However, it is not necessary for the emulsifier or emulsifiers of the aqueous component to be protective agents to practice this invention. Any non-toxic emulsifiers, that are non-inhibitory to cell growth, reproduction and expression of viral, recombinant or natural products, such as, antibodies or native proteins, can be used. Preferably, however, if the emulsifier or emulsifiers of the aqueous component are not protective agents, other protective agents would be included in the media if the culture is to be well-aerated. Such other protective agents would be preferably cell surface stabilizing agents and/or viscosifying agents and/or bubble surface tension reducing agents. Examples of preferred protective agents other than non-toxic polymeric detergents are hydroxyethyl starch, methyl cellulose, carboxymethyl cellulose (as, sodium carboxymethyl cellulose), dextran sulfate, polyvinylpyrrolidone, ficoll, alginic acid and polypropyleneglycol.

Preferred emulsifiers in the lipid solution component are phospholipids and non-toxic, non-ionic polymeric detergents. A preferred phospholipid emulsifier is

lecithin and preferred non-toxic, non-ionic polymeric detergent emulsifiers are polysorbate compounds having the formula:



wherein R is a saturated or unsaturated fatty acid having from 16 to 20 carbons, inclusively;

wherein t is an integer between 10 and 30, inclusively; and

10 wherein u is an integer between 10 and 20, inclusively.

Most preferably, such a polysorbate emulsifier is polyoxyethylene (20) sorbitan monooleate, otherwise known as polysorbate 80. Such a non-toxic, non-ionic polymeric detergent is commercially available as Tween 80 from ICI Americas Inc. (New Murphy Road & Concord Pike, Wilmington, DE 19897, USA). Another  
 15 polysorbate 80 is commercially available as Durfax 80 from Durkee Industrial Foods Group/SCM Corp. (900 Union Commerce Bldg., Cleveland, Ohio 44115, USA). Such a non-ionic, non-toxic, polymeric detergent/emulsifier, such as, polysorbate 80, is preferably present in the lipid solution components of this invention at a concentration from about 0.5 mg/ml to about 75 mg/ml, more preferably from about  
 20 1 mg/ml to about 30 mg/ml, and more preferably from about 2.5 mg/ml to about 25 mg/ml.

Optional ingredients that can be added to the lipid solution component include reducing agent/anti-oxidants, such as, dithiothreitol,  $\beta$ -mercaptoethanol and monothiolglycerol wherein monothiolglycerol is a preferred reducing agent/anti-  
 25 oxidant. Such ingredients are considered amphipathic if they are soluble in both the aqueous and the lipid solution components, and are preferably added to the lipid solution component in appropriate concentrations for the chosen cell line.

Testing whether an emulsifier, a protective agent/emulsifier, combination of emulsifiers, or a combination of a protective agent/emulsifier and an emulsifier or  
 30 emulsifiers is effective in emulsifying the lipids to be supplied to the media of this

invention can be simply performed as follows. First, the candidate emulsifier or combination of emulsifiers must be confirmed as non-toxic to the cells of choice in a manner as described above for candidate protective agents. Secondly, the emulsifying ability of the candidate emulsifier(s) is tested at the determined non-toxic concentrations. The appropriate lipids are dissolved in an appropriate organic solvent, preferably an alcohol ( $C_1$ - $C_3$ ), more preferably ethanol. The candidate emulsifier or each of the combination of emulsifiers is then combined with the lipid solution or in the separate aqueous solution, depending upon in which solution, that is, the organic or aqueous, the candidate emulsifier is more readily combinable. Then the aqueous component is added to the lipid solution component and vigorously agitating as by vortexing. A clear to slightly translucent microemulsion should form if the lipids are successfully emulsified.

For the candidate emulsifier selection test, at least one emulsifier should be present in the lipid solution component and one in the aqueous component. If only one candidate emulsifier is being tested, it can be tested with a known emulsifier, such as, for example, if the candidate emulsifier is readily combinable in water, it could be tested in a candidate aqueous component with Tween 80 as the emulsifier in the lipid solution component; whereas, if the candidate emulsifier is readily combinable in a solution of appropriate lipids, it could be tested with Pluronic F68 in an aqueous component. Another option would be to test a sole candidate emulsifier in both the aqueous and lipid solution components, that is, wherein the same emulsifier would be present at appropriate concentrations in both a candidate aqueous component and a candidate lipid solution component.

One option for emulsifying lipids to be supplied to cell culture media according to this invention is a dual emulsifier system wherein, as noted above, the protective agent is an emulsifier as well as a protective agent and can act in conjunction with an emulsifier or combination of emulsifiers present in the lipid solution component. A preferred example of a dual emulsifier system of the media of this invention is the combination of a protective agent/emulsifier, preferably a Pluronic polyol, more preferably Pluronic F68 or Pluronic F88, and still more preferably Pluronic F68, and a non-toxic non-ionic polymeric detergent, preferably a polysorbate compound, and more preferably polysorbate 80.

Another option for emulsifying lipids according to this invention is a system

wherein the protective agent is not significantly emulsifying but wherein one or more additional emulsifiers are present in the aqueous component which is added to the lipid solution component and act in conjunction with the emulsifiers present therein to form a microemulsion.

- 5       The choice of media to which the lipid microemulsions of this invention are added depends on the particular cells that are being cultured. There are commercially available media for all cell types.

For example, there are a wide variety of commercially available media for insect cell culture which include such commercial available basal medium as, for  
10   example, TC10 without tryptose broth [commercially available from Microbiological Associates; see Gardiner et al., J. Invert. Pathol., 25:363 (1975)], Grace's Antheraea medium [Vaughn et al., TCA Manual, 3(1) (1976); Yunker et al., Science, 155:1565-1566 (1967)], Medium M20 of Mark's [Vaughn et al., TCA Manual, 3(1) (1976); Marks, In Kruse et al. (eds), Tissue Culture Methods and Applications, pp. 153-156  
15 (1973)], Goodwin's IPL-52 Medium [Goodwin, In Vitro, 11:369-378 (1975)], Goodwin's IPL Medium [Goodwin et al., In Kurstak et al. (eds.), Invertebrate Systems In Vitro (1980)], Goodwin's IPL-76 Peptone Medium [Goodwin et al., id.; Goodwin et al., In vitro, 14:485-494 (1978)], Hink's TMH FH Medium (Revised) [Hink, Nature (London), 226:466-467 (1970)], Medium S-301 of Hansen [Hansen, In  
20 Maramorosch (ed.), Invertebrate Tissue Culture Research Applications, pp. 75-99 (1976)]; Vaughn et al, TCA Manual, 3(1) (1976)], and IPL-41 Medium [Weiss et al., In Vitro, 17(6):495-502 (1981)], wherein IPL-41 is a preferred basal medium.

IPL-41 basal medium is commercial available from a number of vendors and is described in Weiss et al., In Vitro, 17(6):495-502 (June 1981) and in Weiss et al.,  
25 CRC Press, supra, pp. 70-72 (1986). Table 1 of Weiss et al. (In Vitro) at page 496, and Table 3 of Weiss et al., CRC Press, at pages 71-72 outline all the components of IPL-41 and provide their proportions in mg/l; said tables are herein incorporated by reference. At page 497 of Weiss et al., (In Vitro), the preparation of the complete medium IPL-41 is described wherein tryptose phosphate broth (TPB) and fetal bovine  
30 serum (FBS) are added. The IPL-41 basal medium employed in preparing the media of this invention preferably does not contain tryptose phosphate broth (TPB) or fetal bovine serum (FBS). PCT US 89/01028, supra, describes preferred replacements for serum and tryptose phosphate broth wherein lipid microemulsions of this invention

supply the lipid fraction in insect culture media.

A "basal medium" is herein defined as a nutrient mixture of inorganic salts, sugars, amino acids, optionally also containing vitamins, organic acids and/or buffers. Basal media together with supplements provide the nutrients necessary to support cell  
5 life, growth and reproduction. The choice of basal media used as the starting point for preparing the media of this invention is not critical, but should be appropriate for the cell line selected and would most preferably contain little or not serum, serum albumin or other lipid carriers. The basal medium can also be considered optional in the sense that appropriate peptone and lipid components can be selected which  
10 provide such necessary nutrients as amino acids and vitamins required to support cell life, growth, reproduction, and expression of recombinant or viral products and production of antibodies.

Commercially available media for mammalian cells include Dulbecco's Modified Eagle Medium (DME; available from Irvine Scientific, Santa Ana,  
15 California USA); RPMI 1640 Medium (also available from Irvine Scientific); HL-I (Ventrex Labs, Portland, Maine USA); HB104 (Hana Biologicals, Berkeley, California USA); Eagle Minimum Essential Medium; and Ham F-12 Medium; RITC 80-7 [Yamane et al., Exp. Cell. Res., 134:470 (1981)]; RITC 56-1 [Yamane et al., In Kohno et al. (eds.), International Symposium on Clinical Potentials and Interferons in  
20 Viral Disease and Malignant Tumors, pp. 355-364.

Examples of media for plant cell culture include those described in Murashige et al., Physiol. Plant, 15:473 (1962); Gamborg et al., Exp. Cell Res., 50:151 (1968); and Gamborg et al., In Vitro, 12:473 (1976).

Further examples of culture media for animal cells, algae, bacteria, fungi and  
25 protozoa can be found in Cote et al. (eds.), ATCC Media Handbook, first edition 1984 (ATCC 1984).

The media of this invention can also include other preferably water-soluble ingredients, for example, insulin to enhance glucose uptake, transferrin for iron transport, trace elements as selenium, catalase as a peroxidation protectant,  
30 ethanolamine as a lipid precursor, steroid hormones as testosterone, thyroid hormone as triiodothyronine, nucleic acid precursors such as hypoxanthine, thymidine, deoxyadenosine, and deoxycytidine, as well as vitamins, minerals, amino acids and other nutrients such as L-glutamine, pyruvate, alpha-glycerol phosphate, glycerol,



folic acid, and inositol which are contained in conventional serum-supplemented or serum free media for cell culture. Although some of such ingredients are proteins, they are not being supplied for the purpose of carrying lipids and are preferably at low concentrations.

5       The method of preparing a culture medium to which the lipid microemulsions of this invention are added is not critical. The media may be prepared, for example, by dissolving all the ingredients and additives in water in their respective appropriate concentrations first and then filtering the solution on a membrane filter under pressure to get a sterilized culture medium.

10       The method of culturing cells with the media of this invention is also not critical. Cells are cultured in the media of this invention under approximately the same conditions as those for conventional culture media, except that whenever possible, cells should be growth under well-aerated conditions, that is, in agitated and/or sparged cultures. For optimal culture conditions for the growth of insect cells  
15 see PCT US 89/01029 and PCT US 89/01028, supra, which are herein incorporated by reference.

In general, insect cells grown in the media of this invention are cultured in a temperature range and under conditions appropriate for the particular cell line selected. For example, Spodoptera frugiperda cells, preferably Sf9 cells, are cultured  
20 in a temperature range of from about 25°C to about 28°C and wherein the pH of the culture medium is preferably maintained in a range from about 6 to about 7, more preferably about 6.2 to about 6.4.

The media of this invention are employable not only for the growth of cells, but also for the production of useful physiologically active substances such as  
25 interferons, lymphokines and antibodies. According to the examples of this invention, recombinant CSF-1 is produced by host insect cells cultured in media containing lipid microemulsions of this invention. "Colony stimulating factor (CSF-1)" refers to a protein which exhibits the spectrum of activity understood in the art for CSF-1, that is, when applied to the standard in vitro colony stimulating assay of Metcalf, J. Cell  
30 Physiol., 76:89 (1970), it results in the formation of primarily macrophage colonies. Native CSF-1 is a glycosylated dimer, dimerization may be necessary for activity. The term CSF-1 herein refers to both dimeric and monomeric forms.

Other heterologous proteins that have been expressed in insect cells via a

baculovirus expression vector system (BEVS) are outlined in Summers et al., Banbury Report: Genetically Altered Viruses in the Environment, 22:319-329 (1985). However, those skilled in the art who have the benefit of this disclosure, will recognize that many other recombinant proteins can be produced by animal, plant and/or microbial cells according to this invention. Exemplary recombinant proteins include, without limitation colony stimulating factors [for example, long and short form CSF-1 or M-CSF, G-CSF, GM-CSF and interleukin-3 among others], modified pro-urokinase or urokinase, tissue plasminogen activator (TPA), TPA-urokinase hybrids, toxic proteins such as whole ricin toxin, ricin A chain, products containing ricin A, as well as, interferons  $\alpha$ ,  $\beta$  and  $\gamma$  and hybrids thereof), interleukins, tumor necrosis factor, erythropoietin and other hematopoietic growth factors, human growth hormone, as well as porcine, bovine and other growth hormones, epidermal growth factor, insulin, hepatitis B vaccine, superoxide dismutase, Factor VIII, Factor VIII C, atrial natriuretic factor, feline leukemia virus vaccines, as, for example, gp70 polypeptides, lectins such as Ricin communis agglutinin (RCA), diphtheria toxin, gelonin, exotoxin from Pseudomonas aeruginosa, toxic proteins from Phytolacca americana (PAPI, PAPII and PAP-s), insecticidal proteins from Bacillus thuringiensis, many enzymes as for example, CAT, as well as innumerable other hybrid proteins.

Further, as indicated above, a variety of monoclonal antibodies can be produced by hybridomas cultured in media of this invention and various viral products can be produced from insect cells so cultured.

Primarily, the media of this invention would be used for suspension cultures including microcarrier cultures. The lipid microemulsions of this invention can however be added to any media to which lipids are considered a beneficial addition. In general, if cells can be grown successfully in media wherein serum, albumin, other proteinaceous lipid carrier or other non-protein lipid carriers, such as a cyclodextrin or mixture of cyclodextrins (see Yamane et al., supra; U.S. Patent No. 4,533,637), are employed, then the cells can be grown in the media of this invention wherein the lipids are supplied as a lipid microemulsion.

The reason lipids are supplied to the cells is not critical. They can be supplied as a microemulsion according to this invention whether they are considered as essential or growth promoting nutrients, physical or chemical protectants or as having other functions, as for example, as a solvolytic agent, as a membrane

modifying agent, as a surface tension reducing agent and/or as a cell surface stabilizing agent.

The cells can be of animal, microbial or plant origin. If animal cells, they can be from vertebrates or invertebrates. Preferably, the cells are those which can produce recombinant, viral and/or natural products. Exemplary vertebrate cells are mammalian cells, for example, lymphocytes, fibroblasts, epithelial cells, ovarian cells, and their transformed cells, various neoplastic cells, and hybridomas derived therefrom. More specifically, examples of mammalian cells include Chinese hamster ovarian cells, Epstein-Barr Virus (EBV)-transformed human lymphoblastoid cell lines such as UMCL and C51804, human Burkitt's lymphoma-derived Namalwa cells, murine lymphoid cell-derived myeloma SPI cells, human fibroblast cells such as HEL and IMR-90, human tumor-derived epithelial cells such as HeLa-S<sub>3</sub>, Hep-2 and KB, human primary cultured cells, rat Yoshida sarcoma cells, hamster fibroblast cells BHK-21, murine fibroblast cells 3T3, murine lymphoma cells YAC-1, human/mouse hybridomas such as stable cell line D-234-4-27-8 which produces anti-LPS IgM (deposited at the American Type Culture Collection, Rockville, Maryland USA on August 10, 1984 under ATCC Accession No. HB 8598) and hybridomas which produce monoclonal antibodies to human fibroblast interferon.

Exemplary invertebrate cells are insect cells, preferably cells which can produce viral or recombinant products upon infection, respectively, with either wild-type viruses or recombinant baculoviruses and which have been shown to grow, reproduce and express recombinant and/or viral products in a medium containing serum, albumin, another protein and/or other lipid carriers. For example, insect cells that can be grown in IPL-41 basal medium containing approximately 10% serum, can be grown in media wherein lipids are supplied as microemulsions according to this invention. Such insect cell lines include Bombyx mori, Lymantria dispar, Trichoplusia ni and Spodoptera frugiperda. [See generally, Granados et al. (eds.), The Biology of Baculoviruses (CRC Press 1986); Vaughn, Adv. Cell. Cult., Maramorosch (ed.), Invert. Tissue Culture: Research Applics., p. 295 (1976); and Vaughn, In, Barigozzi (eds.) Proceedings of Internatl. Colloq. Invert. Tissue Culture, (2nd, Tremezzo, 1967), p. 119 (1968).]

Further, insect cells that can be grown in the media of this invention are preferably from any order of the Class Insecta which can be hosts to a baculovirus.

expression vector system, or other wild-type viruses, but are preferably from the Diptera or Lepidoptera orders. About 300 insect species have been reported to have nuclear polyhydrosis virus (NPV) diseases, the majority (243) of which were isolated from Lepidoptera. [Weiss et al., "Cell Culture Methods for Large-Scale Propagation of Baculoviruses," In Granados et al. (eds.), The Biology of Baculoviruses: Vol. II Practical Application for Insect Control, pp.63-87 at p. 64 (1986).] Insect cell lines derived from the following insects are exemplary: Carpocapsa pomonella preferably cell line CP-128; Trichoplusia ni (preferably cell line TN-368); Autographa californica; Spodoptera frugiperda (preferably cell line Sf9); Lymantria dispar;

10 Mamestra brassicae; Aedes albopictus; Orgyia pseudotsugata; Neodiprion sertifer; Aedes aegypti; Antheraea eucalypti; Gnorimoschema operculella; Galleria mellonella; Spodoptera littoralis; Blatella germanica; Drosophila melanogaster; Heliothis zea; Spodoptera exigua; Rachiplusia ou; Plodia interpunctella; Amsacta moorei; Agrotis c-nigrum, Adoxophyes orana, Agrotis segetum, Bombyx mori, Hyponomeuta malinellus,

15 Colias eurytheme, Anticarsia gemmatia, Apanteles melanoscelus, Arctia caja, and Porthetria dispar. Preferred insect cells are from Spodoptera frugiperda, and especially preferred is cell line Sf9. The Sf9 cell line used in the examples herein was obtained from Max D. Summers (Texas A & M University, College Station, TX 77843 USA). Other S. frugiperda cell lines, such as IPL-Sf21AE III, are described in

20 Vaughn et al., In Vitro, 13:213-217 (1977).

The preferred insect cell lines cultured in the media of this invention are suitable for the reproduction of numerous insect-pathogenic viruses such as parvoviruses, pox viruses, baculoviruses and rhabdoviruses, of which nucleopolyhydrosis viruses (NPV) and granulosis viruses (GV) from the group of

25 baculoviruses are preferred. Further preferred are NPV viruses such as those from Autographa spp., Spodoptera spp., Trichoplusia spp., Rachiplusia spp., Galleria spp. and Lymantria spp. More preferred are baculoviruses strains Autographa californica NPV (AcNPV), Rachiplusia ou NPV, Galleria mellonella NPV and any plaque-purified strains of AcNPV, such as E2, R9, S1, M3, characterized and described by

30 Smith et al., J. Virol., 30:828-838 (1979); Smith et al., J. Virol., 33:311-319 (1980); and Smith et al., Virol., 89:517-527 (1978).

European Patent Application 127,839 (published December 12, 1984) to Smith et al. describes a method for producing a recombinant baculovirus expression vector,

capable of expressing a selected gene in a host insect cell. Said European application is herein incorporated by reference. The recombinant baculovirus expression vector is cotransfected with wild-type baculovirus DNA into a host insect cell, wherein recombination occurs. Recombinant baculoviruses are then detected and isolated according to methods described in EP 127,839 and Summers et al., "A Manual and Methods for Baculovirus Vectors and Insect Cell Culture Procedures," Texas Agricultural Experiment Bulletin No. 1555 (Texas A & M University; May 1987). The resultant recombinant baculovirus is then used to infect cultured insect cells and the protein product from the incorporated selected gene is expressed by the insect cells and secreted into the medium. Exemplified therein is the production of recombinant  $\beta$ -interferon, interleukin-2, and chloramphenicol acetyltransferase (CAT) via the culturing of *S. frugiperda* cells infected with a recombinant AcNPV expression vector into the genome of which the appropriate gene had been inserted. Further information concerning such a recombinant baculovirus expression system and its use in expression recombinant proteins can be found in Summers et al., *id.*

PCT application, Nos. US 89/01038, US 88/05081 and US 89/01037 describe, respectively, the expression at high levels of biologically active recombinant colony stimulating factors, modified pro-urokinase, urokinase and novel hybrid proteins thereof, and ricin toxin proteins, wherein an insect cell/baculovirus expression system is employed to produce such proteins.

PCT US 89/01038, *supra*, specifically describes the construction of baculovirus CSF-1 expression and transfer vectors, including pAcM4 and pAcM6, used to prepare the recombinant baculoviruses AcM4 and pAcM6 by cotransfection with baculovirus DNA in Sf9 cells. Said recombinant baculovirus transfer vectors pAcM4 and pAcM6 referred to in the examples below has been deposited at the American Type Culture Collection (ATCC) [12301 Parklawn Drive, Rockville, MD 20852 (USA)] on June 12, 1987 in *E. coli*/MM294 under the respective designations ATCC Nos. 67429 and 67438. Said vectors have also been deposited and are maintained in the Cetus Master Culture Collection (CMCC) under the respective designations CMCC No. 3002 and CMCC No. 2996. Recombinant baculovirus AcM4 carries a nucleotide sequence which encodes for a 150 amino acid form of rCSF-1 whereas the recombinant baculovirus AcM6 carries a nucleotide sequence which encodes for a 522 amino acid form of rCSF-1.

The effect of timing of the infection of the insect cells with a recombinant baculovirus has been shown to be critical for enhanced specific productivity. The specific production of the recombinant protein was found to be constant during the exponential phase of cell growth under non-oxygen limited conditions. Late  
5 infection, under non-exponential growth conditions, resulted in lower specific productivity and lower final titer. It is preferred that the exponential growth phase be extended to the highest possible cell densities to achieve the highest total productivity of the recombinant protein product. Infection of the host insect cells under conditions that limit growth, for example, in the stationary phase of cell growth,  
10 results in a reduced specific productivity of the recombinant protein product.

Specific productivity (U or mg/cell) of the recombinant protein product is relative independent of cell density at the time of infection as long as the culture is in exponential growth. For example, when Spodoptera frugiperda cells are the host insect cells and when preferred media of this invention as described for insect cells in  
15 PCT US 89/01028, supra, are used, cell densities of from about  $1.0 \times 10^6$  to about  $4.0 \times 10^6$  cells/ml are preferred for infection with the recombinant baculovirus, more preferably from about  $2.5 \times 10^6$  to about  $3.5 \times 10^6$  cells/ml.

When employing the baculovirus expression vector system, the timing of the harvest of the recombinant protein product is critical to avoid contamination of the  
20 recombinant protein by viral and cell lysis proteins and to simplify thereby the downstream purification of the recombinant protein. With considerations for the stability of the product, it would be preferred to harvest the recombinant product before significant cell lysis has occurred.

Further, each recombinant, viral or native product expressed in the media of  
25 this invention should be checked for stability and degradation over the course of the fermentation run. Such considerations should enter into a determination of the optimal harvest time.

The following examples further illustrate the aqueous and lipid solution components, the lipid microemulsions, media and methods of this invention. These  
30 examples are not intended to limit the invention in any manner.

Example 1Lipid Microemulsion Preparation for Mammalian Cells

10 mg of cod liver oil, 4.5 mg of cholesterol, 2.0 mg of alpha-tocopherol acetate and 25 mg of Tween 80 were added to 10 ml of ethanol, dissolved therein, and the solution was filter sterilized. To prepare a quantity of lipid microemulsion sufficient for approximately one liter of mammalian culture medium, 10 ml of 10% Pluronic F68 in water was slowly and aseptically added with agitation by vortexing to 1 ml of the lipid solution component. A microemulsion was thereby formed which can then be added to a culture medium that has already been filter sterilized.

Example 2Lipid Microemulsion Preparation for Mammalian Cells

10 mg of monothiolglycerol, 10 mg of linoleic acid, 10 mg of lecithin, 5 mg of cholesterol, 5 mg of alpha-tocopherol acetate, and 20 mg of Tween 80 were added to 10 ml of ethanol and dissolved therein. To prepare a quantity of lipid microemulsion sufficient for approximately 1 liter of mammalian culture medium, 10 ml of 10% Pluronic F68 in water was slowly added with agitation by vortexing to 1 ml of the lipid solution component. A lipid microemulsion was thereby formed which can be then added to a culture medium which medium can then be filter sterilized without a concern of loss of lipids during the process. This emulsion is referred to as the A7 emulsion.

Example 3Lipid Microemulsion Preparation for Insect Cells

100 mg of cod liver oil, 250 mg of Tween 80, 45 mg of cholesterol and 20 mg of alpha-tocopherol acetate were added to 10 ml of ethanol and dissolved therein. The lipid solution component was then filter sterilized.

To prepare a quantity of lipid microemulsion sufficient for about 1 liter of insect culture medium, 10 ml of 10% Pluronic F68 was slowly and aseptically added with agitation by vortexing to 1 ml of the lipid solution component. A microemulsion was thereby formed which can then be added to a insect cell culture medium that has already been filter sterilized.

Example 4Insect Cell Growth in Serum-Free Media  
Containing Lipid Microemulsion

50 ml cultures of Sf9 cells were grown in 250 ml shake flasks agitated at 100-150 rpm (within an orbital radius of one-half inch) at 27°C, in both ISFM-3 and ISFM-4 media for a number of passages. Said media contain the lipid microemulsion described above in Example 3. Initially, the ISFM-4 culture had a longer lag phase than the ISFM-3 culture, but by the third passage, both cultures grew equally well with short lag phases. Both ISFM-3 and ISFM-4 cultures have longer lag phases than conventional serum containing insect culture media. The average growth characteristics of eight passages in both media are shown in Table 2 below. These media provide growth rates ( $T_d=17-24$  hours) maximum cell densities of about  $5 \times 10^6$  cells/ml and cell viabilities (99-100%) which are comparable to those found in 10% serum containing media under similar culture conditions.

TABLE 2

<u>Lag Phase*</u> <u>(hours)</u>		<u>Population Doubling</u> <u>Time (hours)</u>	<u>Maximum Cell Densit</u> <u>(<math>10^6</math> cells/ml)</u>
ISFM-3	14	22	5.0
ISFM-4	15	22	6.1

\* Lag phase was calculated by extrapolating the exponential phase plot (semi-log) back to the initial cell density ( $1 \times 10^5$  cells/ml. The x-intercept hours of culture incubation) is equal to the lag phase.

A second experiment was conducted to show the effect of the liquid supplement on the growth of Sf9 cells in ISFM-4 serum free media. After two passages in serum free media, the cells were seeded in serum free media with or without the lipid microemulsion of Example 3, and cell growth monitored. It is apparent from figure 1 that the microemulsion is required for Sf9 cell growth.



Example 5Production of Recombinant CSF-1 by Insect  
Cells Grown in Serum-Free Media Containing  
Lipid Microemulsion

5 Sf9 cells grown for 25-30 passages (75-120 generations) according to the methods described in Example 4, above, were tested for production of rCSF-1 via a baculovirus expression vector system. A Sf9 culture in IPL-41 complete medium with tryptose phosphate broth (2.6 g/l) and 10% fetal bovine serum was employed as a control. An additional medium to those used in Example 4, that is, SFM2M  
10 without BSA as described in the Detailed Description above was also used in this example.

The recombinant baculovirus AcM4 was used to infect exponentially growth cultures at a multiplicity of infection of 1. As shown in Table 3, below, the serum containing culture and the serum free cultures which contain lipid microemulsions of  
15 this invention produced similar levels of rCSF-1 (about  $10^6$  U/ml) as estimated by RIA.

TABLE 3

rCSF-1 Production by Sf9 Cells Growing in Various Media  
at 5 Days After Infection with AcM4

20	<u>Media</u>	<u>Cell Density at Infection (<math>10^6</math> cells/ml)</u>	<u>rCSF-1 Produced by 5 Days Postinfection (<math>10^5</math> RIA U/ml)</u>
	10% Serum*	3.2	9.1
	SFM2M without BSA	2.5	10.2
25	ISFM-3	3.1	10.1
	ISFM-4	3.0	9.9

\* 10% serum is IPL-41 complete medium with TPB and FBS.

Example 6Insect Cells Adapt Readily From Serum Containing Media  
to Serum Free Media Containing Lipid Microemulsion

Sf9 cells maintained in 10% serum containing medium, that is, IPL-41  
5 complete medium, readily adapted to SFM2M without BSA, ISFM-3 and ISFM-4  
upon the first and second passages. Subsequent passages and infections with the  
recombinant baculovirus AcM4 and a second recombinant baculovirus AcM6 have  
confirmed that such media containing lipid microemulsions are suitable media for the  
large-scale growth of insect cells and for the production of recombinant protein  
10 products via a recombinant baculovirus expression vector system.

Example 7Growth of CHO Cells

The lipid microemulsions can be formulated in a wide variety of media, and  
are not restricted to insect cell media nor to culture of insect cells. CHO cells  
15 transformed with the M-CSF expression vector CHO/17/150, described in PCT  
application No. US 89/02746, published April 6, 1989, were seeded at an initial  
density of  $1 \times 10^5$  and grown for 14 days, after which the media was assayed using  
standard radioimmune assay procedures. The lipid microemulsion shown in Example  
1 was used to supplement media consisting of a 1:1 mixture of MCDB 301,  
20 described by Hamilton and Ham, in *In Vitro* 13:(9) 537, 1977; and DME-spinners,  
plus 4 mM glutamine, 10  $\mu$ g/ml insulin, 10  $\mu$ g/ml transferrin, 10 mg/ml ethanolamine,  
and 400  $\mu$ g/ml bovine serum albumin. Cells were grown both in serum free media,  
or media supplement with 1% fetal bovine serum. Table 4 shows that in serum  
containing media, CHO cells attain a density of about  $1.3 \times 10^6$  cells/ml, and  
25 expresses m-CSF at a level of about 310,000 U/ml. In serum free media, a cell  
density of about  $0.9 \times 10^6$  cells/ml is reached, and CHO cells in this media express  
about 240,000 U/ml of m-CSF.

Table 4

Cell Line	Product	Serum-Supplemented		Serum-Free + Lipid Emulsion			
		Cell Density ( $\times 10^6$ /ml)	Product Level	Medium	Cell Density ( $\times 10^6$ /ml)	Product Level (mg/l)	Medium
Sf9	Recombinant Hu M-CSP	5	37mg/l	ISPM4 + 10ZFBS	5	40mg/l	ISPM4
CHO	Recombinant Hu M-CSP	1.3	310,000 /ml	BHCHO + 10ZFBS	0.9	240,000 /ml	BHCHO
NAHALWA	---	6.8	---	DM8+20Z1PL/41 + 10ZFBS	3.7	---	DM8+20Z1PL/41
MOPC21 Hu MYELOMA	Hu IgG	nd	nd	---	3	100mg/l	DM8+20Z1PL/41

ISFM4: IPL/41, 4g/l yeastolate, insect lipids.

BHCHO: 1:1 mix MCD8301 and DME-Spinners, 4mM glutamine, 10ug/ml insulin, 10 ug/ml transferrin, 10mg/ml, ethanolamine, 400 mg/ml BSA, insect lipid mix (1/10 concentration).

DM8+20%IPL/41: adME-HP, RPMI 1640 and IPL/41 (4:4:2). Also 5 ug/ml insulin, 5 ug/ml transferrin, 5 ng/ml selenium, 8 mM glutamine, 10 mg/ml ethanolamine and A7 lipids.

### Example 8

To further establish that the lipid microemulsions shown herein support the growth and product expression of other mammalian cell types, several cell lines of immunologic origin, as well as the Namalwa cell line, were cultured. Table 4 shows that the cell lines MOPC21 and Namalwa respond favorable to media supplement  
5 with the A7 lipid microemulsion.

The medium consisted of a 1:1 mixture of DM8 and IPL/41, plus the A7 lipid microemulsion of Example 2. DM8 was formulated from sDME-HP, RPMI 1640, and IPL/41 (4:4:1). Also added were insulin (5 µg/ml), transferrin (5 ng/ml), selenium  
10 (5 µg/ml), glutamine (8 mM), and 10 mg/ml ethanolamine. An ELISA assay to determine antibody levels was run.

### Example 9

#### Characterization of Lipid Microemulsion Particle Size Before and After Sterile Filtration

One of the advantages of the instant lipid microemulsions is that they are filter sterilizable alone or when combined with media. This is not true of prior art lipid compositions which are not readily filter sterilized with culture media because an indeterminate amount of lipid is not filtered, and remains trapped on the filter. Thus experiments were done to determine the particle size properties of the  
20 microemulsions which favor their filtration.

Low angle laser light scattering measurements were made using a Coulter model N4M laser light scattering instrument and version 10.0W software. A7 lipid mix was prepared using standard procedures and was then diluted (1:5) in DBM medium (dilution to this concentration was required to be in the range appropriate for the sensitivity of the instrument). Measuring intensity at a scattering angle of 90°, a major peak corresponding to particles of size about 95 nm was identified, with less intense peaks at about 9 and 890 nm (Figure 2). The A7 lipid mix was then filter sterilized through a 0.45 µm filter (Sybron Nalge, Rochester NY, No. 120-0045) and the measurement repeated. Peaks corresponding to similar particle sizes (78, 6 and  
30 860 nm) were again detected. Because one does not expect lipids with the particle size distribution observed, particularly those over 800 nm, to readily filter through an 0.45 µm filter, it is thought that the microemulsion distributes on filtration into

smaller particles which are filterable and then reassociate.

### Deposit

As mentioned above, the recombinant baculovirus transfer vectors pAcM4 and pAcM6 in E. coli/MM294 have been deposited at the American Type Culture Collection (ATCC), 12001 Parklawn Drive, Rockville, MD 20852 (USA) on June 12, 1987 under the respective ATCC Nos. 67429 and 67428. Further, the hybridoma cell line D-234-4-27-8 was deposited at the ATCC on August 10, 1984 under Accession No. HB 8598.

Said deposits were made pursuant to a contract between the ATCC and the assignee of this patent application. The contract with the ATCC provides for permanent availability of said strains and progeny thereof upon issuance of a U.S. patent related to this application describing and identifying the deposits or upon the publication or laying open to the public of any U.S. or foreign patent application, whichever comes first, and for the availability of the strains and the progeny thereof to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC §122 and the Commissioner's rules pursuant thereto (including 37 CFR §1.14 with particular reference to 886 OG 638). The assignee of the present application has agreed that if the strains on deposit should die or be lost or destroyed when cultivated under suitable conditions, they will be promptly replaced upon notification with a viable culture of the same strain.

The deposits under the terms of the Budapest Treaty assure that said cultures deposited will be maintained in a viable and uncontaminated condition for a period of at least five years after the most recent request for the furnishing of a sample of the deposited microorganism was received by the ATCC and, in any case, for a period of at least 30 years after the date of the deposit.

Availability of the deposited strains is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

Also, the present invention is not to be considered limited in scope by the deposited recombinant transfer vectors, since the deposited vectors are intended only to be illustrative of particular aspects of the invention. Any recombinant baculoviruses which can function to infect a host insect cell to produce a recombinant

protein product is considered to be within the scope of this invention. Further, various modifications of the invention in addition to those shown and described herein apparent to those skilled in the art from the preceding description are considered to fall within the scope of the appended claims.

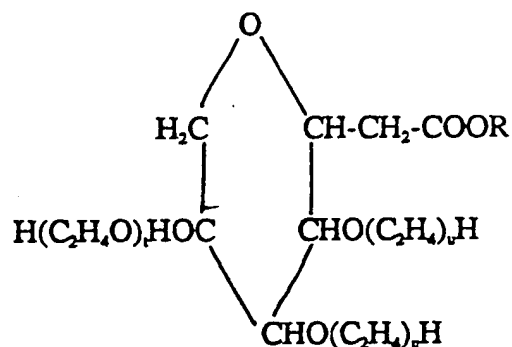
## WHAT IS CLAIMED IS:

1. Cell culture media wherein lipids are supplied in the form of a microemulsion.

2. Cell culture media according to claim 1 wherein the lipid emulsion is formed by combining, with agitation, an aqueous component which comprises one or more emulsifiers in water, and a lipid solution component which comprises one or more lipids and one or more emulsifiers in an organic solvent.

3. Cell culture media according to claim 2 wherein the emulsifiers are non-toxic polymeric detergents and/or phospholipids.

4. Cell culture media according to claim 3 wherein the emulsifiers are phospholipids and/or non-toxic, non-ionic polymeric detergents selected from the group comprising block copolymers of propylene oxide and ethylene oxide and polysorbate compounds having the formula:



wherein R is a saturated or unsaturated fatty acid having from 16 to 20 carbons, inclusively;

wherein t is an integer between 10 and 30, inclusively; and

wherein u is an integer between 10 and 20, inclusively.

5. Cell culture media according to claim 2 wherein the emulsifier or one or more of the emulsifiers of the aqueous component is or are also protective agents.

6. Cell culture media according to claim 5 wherein the emulsifier/protective agent(s) of the aqueous component is or are non-toxic, non-ionic polymeric detergents selected from the group comprising block copolymers of propylene oxide and ethylene oxide.

5        7. Cell culture media according to claim 6 wherein the total concentration (weight/volume) of the Pluronic polyols in the media is from about 0.01% to about 1%.

8. Cell culture media according to claim 7 wherein the total concentration is from about 0.05% to about 0.5%.

10       9. Cell culture media according to claim 8 wherein the total concentration is about 0.1%.

10. Cell culture media according to claim 7 wherein the Pluronic polyols are selected from the group comprising Pluronic F68, F77, F88 and F108.

15       11. Cell culture media according to claim 10 wherein the Pluronic polyols are selected from the group comprising Pluronic F68 and F88.

12. Cell culture media according to claim 11 wherein the emulsifier of the aqueous component is Pluronic F68.

20       13. Cell culture media according to claim 4 wherein one or more phospholipids and/or one or more polysorbate compounds is or are the emulsifier or emulsifiers within the lipid solution component.

14. Cell culture media according to claim 12 wherein the emulsifier(s) of the lipid solution component is or are polyoxyethylene (2) sorbitan monooleate and/or lecithin.



15. Cell culture media according to claim 14 wherein the emulsifier of the lipid solution component is polyoxyethylene (2) sorbitan monooleate, the concentration of which in said lipid solution component is from about 0.5 mg/ml to about 75 mg/ml and wherein the Pluronic F68 of the aqueous component is at a  
5 concentration (weight/volume) of from about 1% to about 10%.

16. Cell culture media according to claim 15 wherein the concentration of the polyoxyethylene (20) sorbitan monooleate in the lipid solution component is from about 1 mg/ml to about 30 mg/ml, and wherein the concentration of the Pluronic F68 in the aqueous component is from about 5% to about 10%.

10 17. Cell culture media according to claim 16 wherein the concentration of the polyoxyethylene (20) sorbitan monooleate in the lipid solution component is from about 2.5 mg/ml to about 25 mg/ml, and wherein the concentration of the Pluronic F68 in the aqueous component is about 10%.

18. Cell culture media wherein serum, albumin or other proteinaceous lipid  
15 carrier is partially replaced with at least one lipid nutrient in the form of a microemulsion.

19. Cell culture media according to claim 2 wherein both the aqueous component and the lipid solution component are filter sterilized before being combined aseptically with agitation to form a lipid microemulsion.

20 20. Cell culture media according to claim 1 wherein the media is filter sterilized after the lipid microemulsion is added thereto.

21. A lipid microemulsion prepared by combining with agitation an aqueous component containing one or more emulsifiers and a lipid solution component containing one or more emulsifiers.

22. A lipid microemulsion component according to claim 21 wherein the lipids are selected from the group comprising unsaturated fatty acids, mixtures of polyunsaturated fatty acid methyl esters, lipid soluble vitamins and sterols, wherein the organic solvent is a C<sub>1</sub>-C<sub>3</sub> alcohol and wherein the emulsifier or emulsifiers are  
5 selected from the group comprising phospholipids and non-ionic, non-toxic polymeric detergents.

23. A lipid microemulsion component according to claim 22 wherein the lipids are selected from the group comprising linoleic acid, linolenic acid, oleic acid; fish liver oil; vitamins A, D and E; and cholesterol;  
10 wherein the organic solvent is ethanol; and  
wherein the emulsifier or emulsifiers are selected from the group comprising lectin and polysorbate compounds.

24. A lipid microemulsion component according to claim 23 further comprising a reducing agent/anti-oxidant.

15 25. A lipid microemulsion component according to claim 24 wherein the reducing agent/anti-oxidant is monothiolglycerol.

26. A lipid microemulsion component according to claim 22 which is stored under nitrogen at a temperature range of from about -20°C to about -80°C.

20 27. A lipid microemulsion component according to claim 22 wherein said component comprises three subcomponents having particle diameters of about 6, 78 and 860 nm.

28. A method of dispersing lipids within a cell culture medium which comprises preparing a lipid microemulsion according to claim 2 and adding said lipid microemulsion to the medium.

25 29. A method of growing cells comprising contacting said cells with the cell culture media of claim 1.

30. A method of growing cells which growth is enhanced by lipids, comprising contacting said cells with the cell culture media of claim 17.

31. A method of growing cells as described in claim 29 wherein said cells are insect or mammalian cells.

5 32. A method of growing cells as described in claim 31 wherein said mammalian cells are antibody producing cells.

33. A method of growing cells as described in claim 30 wherein said cells are insect or mammalian cells.

10 34. A method of growth cells as described in claim 33 wherein said mammalian cells are antibody producing cells.

35. A method of dispersing lipids within a cell culture medium which comprises preparing a lipid microemulsion according to claim 2 and adding said lipid microemulsion to the medium.

36. Cell culture media comprising the lipid solution of claim 27.

15 37. A lipid microemulsion component according to claim 22 wherein said component comprises three subcomponents having particle diameters of about 25 nm, 280 nm, and 1780 nm.

38. Cell culture media comprising the lipid microemulsion of claim 37.

1 / 2

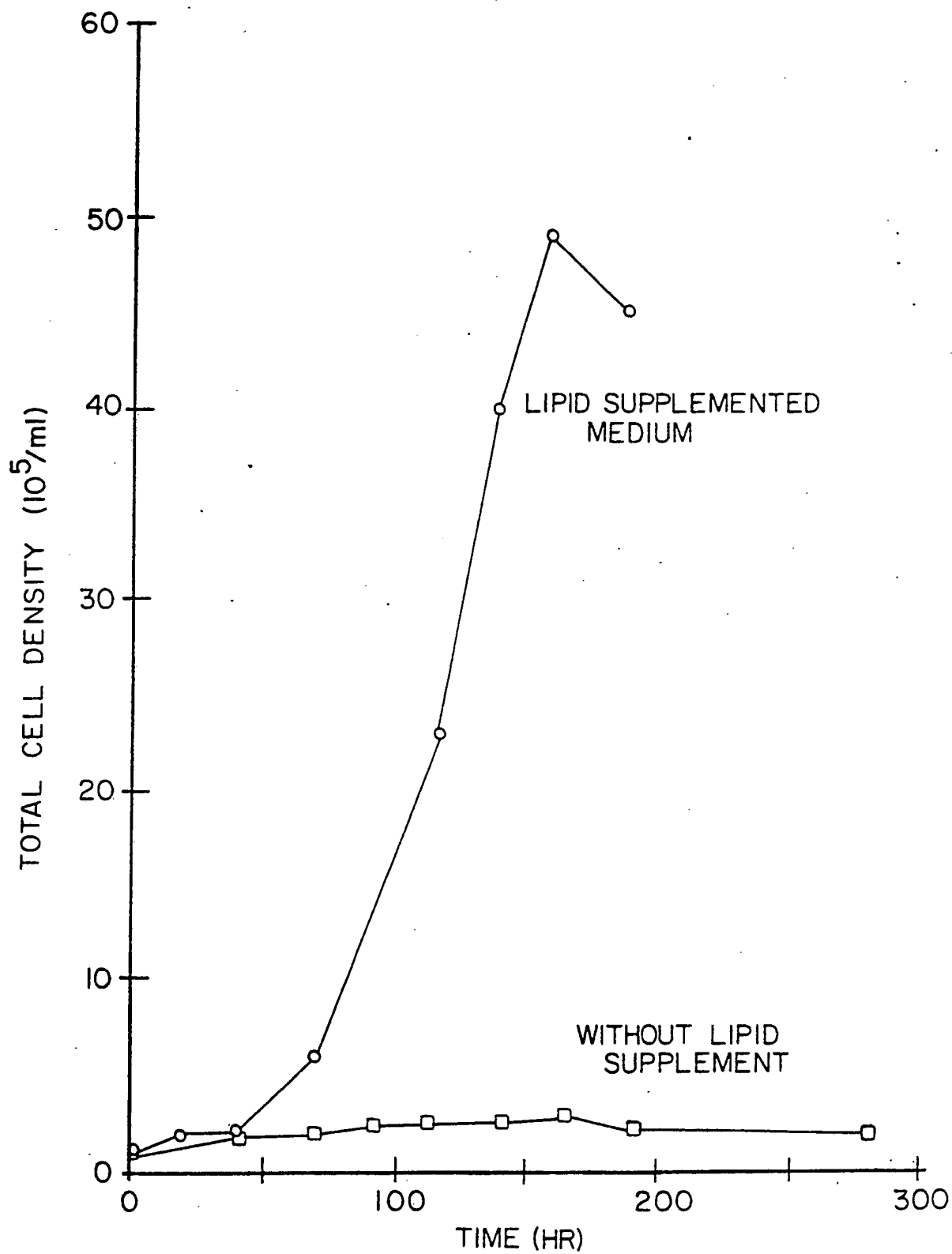
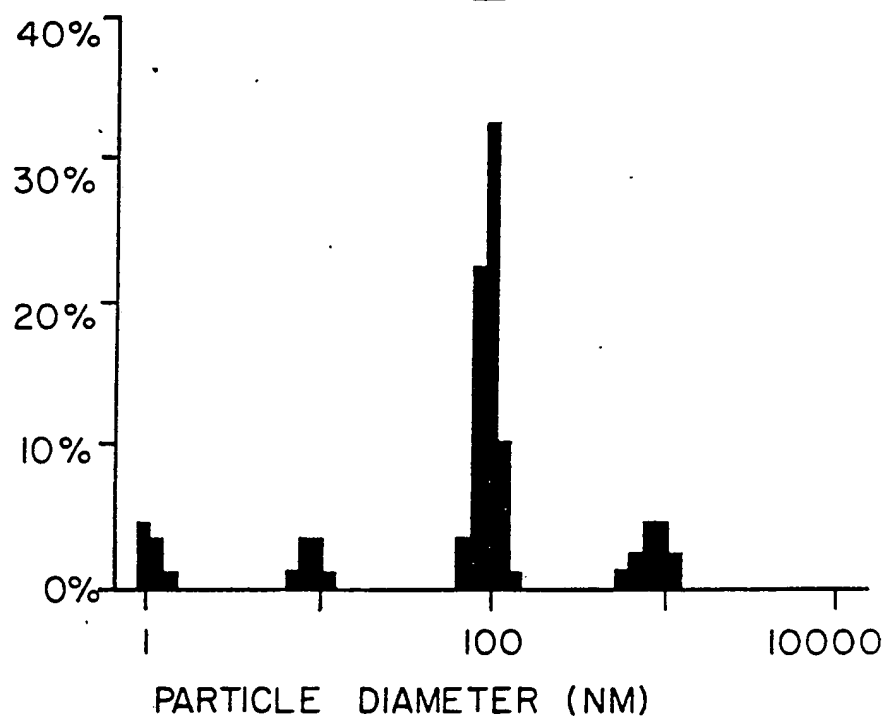


FIG. I

2 / 2

FIG. 2



# INTERNATIONAL SEARCH REPORT

International Application No PCT/US 89/03985

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup> According to International Patent Classification (IPC) or to both National Classification and IPC <div style="font-family: monospace; font-size: 1.2em;">IPC<sup>5</sup>:    C 12 N 5/00</div>																	
<b>II. FIELDS SEARCHED</b> <div style="text-align: center; font-size: 0.8em;">Minimum Documentation Searched <sup>7</sup></div> <table style="width: 100%; border: none;"> <tr> <td style="width: 25%; border: none;">Classification System</td> <td style="width: 75%; border: none;">Classification Symbols</td> </tr> <tr> <td style="border: none; vertical-align: top;">IPC<sup>5</sup></td> <td style="border: none; vertical-align: top;">C 12 N</td> </tr> </table> <div style="text-align: center; font-size: 0.8em; margin-top: 10px;">Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched <sup>8</sup></div>			Classification System	Classification Symbols	IPC <sup>5</sup>	C 12 N											
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<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> <sup>9</sup> <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%; font-size: 0.8em;">Category <sup>10</sup></th> <th style="width: 70%; font-size: 0.8em;">Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup></th> <th style="width: 20%; font-size: 0.8em;">Relevant to Claim No. <sup>13</sup></th> </tr> </thead> <tbody> <tr> <td style="vertical-align: top;">P, X, L</td> <td style="vertical-align: top;">WO, A, 89/01027 (CETUS CORP.) 9 February 1989, see the whole document, esp. claims (art. 4c4 of the Stockholm Act of the Paris Convention) --</td> <td style="vertical-align: top;">1-38</td> </tr> <tr> <td style="vertical-align: top;">X</td> <td style="vertical-align: top;">Experimental Cell Research, vol. 172, 1987 P.A. Ryan et al.: "Modification of MCDB 110 medium to support prolonged growth and consistent high cloning efficiency of diploid human fibroblasts", pages 318-328, see page 320, lines 1-17; pages 321, 322</td> <td style="vertical-align: top;">1-3, 18-29, 31, 35</td> </tr> <tr> <td style="vertical-align: top;">Y</td> <td style="vertical-align: top;">--</td> <td style="vertical-align: top;">14, 30-36</td> </tr> <tr> <td style="vertical-align: top;">Y</td> <td style="vertical-align: top;">Proc. Natl. Acad. Sci. USA, vol. 78, no. 9, September 1981 W.J. Bettger et al.: "Rapid clonal growth and serial passage of human diploid fibroblasts in a lipid-enriched synthetic medium supplemented with epidermal growth factor, insulin,</td> <td style="vertical-align: top;">14, 30-36</td> </tr> </tbody> </table>			Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>	P, X, L	WO, A, 89/01027 (CETUS CORP.) 9 February 1989, see the whole document, esp. claims (art. 4c4 of the Stockholm Act of the Paris Convention) --	1-38	X	Experimental Cell Research, vol. 172, 1987 P.A. Ryan et al.: "Modification of MCDB 110 medium to support prolonged growth and consistent high cloning efficiency of diploid human fibroblasts", pages 318-328, see page 320, lines 1-17; pages 321, 322	1-3, 18-29, 31, 35	Y	--	14, 30-36	Y	Proc. Natl. Acad. Sci. USA, vol. 78, no. 9, September 1981 W.J. Bettger et al.: "Rapid clonal growth and serial passage of human diploid fibroblasts in a lipid-enriched synthetic medium supplemented with epidermal growth factor, insulin,	14, 30-36
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<div style="font-size: 0.8em;"> <div style="display: flex; justify-content: space-between;"> <div style="width: 48%;"> <p><sup>10</sup> Special categories of cited documents: <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 48%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> </div> </div> </div>																	
<b>IV. CERTIFICATE</b> <sup>14</sup> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; padding: 5px;">           Date of the Actual Completion of the International Search   <div style="font-size: 1.2em;">8th December 1989</div> </td> <td style="width: 50%; padding: 5px;">           Date of Mailing of this International Search Report   <div style="font-size: 1.2em;">06.02.90</div> </td> </tr> <tr> <td style="width: 50%; padding: 5px;">           International Searching Authority   <div style="text-align: center;">EUROPEAN PATENT OFFICE</div> </td> <td style="width: 50%; padding: 5px;">           Signature of Authorized Officer  <div style="text-align: center;">C.D. v.d. Vliet </div> </td> </tr> </table>			Date of the Actual Completion of the International Search  <div style="font-size: 1.2em;">8th December 1989</div>	Date of Mailing of this International Search Report  <div style="font-size: 1.2em;">06.02.90</div>	International Searching Authority  <div style="text-align: center;">EUROPEAN PATENT OFFICE</div>	Signature of Authorized Officer <div style="text-align: center;">C.D. v.d. Vliet </div>											
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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
	and dexamethasone", pages 5588-5592, see tables 1 and 2 --	
Y	US, Re. 30 985 (O.A. CARTAYA) 29 June 1982, see abstract; column 7 --	1-22
Y	Journal of Clinical Microbiology, vol. 2, no. 1, July 1975 A. Mizrahi: "Pluronic polyols in human lymphocyte cell line cultures", pages 11-13, see page 11 --	1-22
Y	Development. Biol. Standard, vol. 55, 1984, S. Karger, (Basel, CH) A. Mizrahi: "Oxygen in human lympho- blastoid cell line cultures and effect of polymers in agitated and aerated cultures", pages 93-102, see table I  -----	1-22

# ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 8903985

SA 31166

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.  
The members are as contained in the European Patent Office EDP file on 29/01/90  
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 8901027	09-02-89	AU-A- 2255488	01-03-89
US-E- 30985	29-06-82	US-A- 4205126	27-05-80